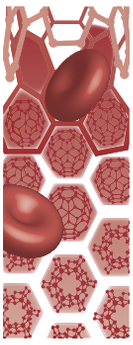
Research Article

Nanomedicine



Functionalized core/shell nanofibers for the differentiation of mesenchymal stem cells for vascular tissue engineering

Hariharan Ezhilarasu[1], Asif Sadiq[1], Greeshma Ratheesh[2], Sreepathy Sridhar[3], Seeram Ramakrishna[1], Mohd Hasbi Ab. Rahim[4], Mashitah M Yusoff[4], Rajan Jose[4] & Venugopal Jayarama Reddy*[,1,4]

[1]Department of Mechanical Engineering, Center for Nanofibers & Nanotechnology, Faculty of Engineering, National University of Singapore, Singapore

[2]Institute of Health & Biomedical Innovation, Science & Engineering Faculty, Queensland University of Technology (QUT), Australia

[3]Department of Mechanical & Construction Engineering, Northumbria University, Newcastle upon Tyne, NE1 8ST, UK

[4]Faculty of Industrial Sciences & Technology, Universiti Malaysia Pahang, 26300 Gambang, Kuantan, Pahang, Malaysia

*Author for correspondence: Tel.: +60 10 951 8246; venugopal@ump.edu.my

Aim: Atherosclerosis is a common cardiovascular disease causing medical problems globally leading to coronary artery bypass surgery. The present study is to fabricate core/shell nanofibers to encapsulate VEGF for the differentiation of mesenchymal stem cells (MSCs) into smooth muscle cells to develop vascular grafts. **Materials & methods:** The fabricated core/shell nanofibers contained polycaprolactone/gelatin as the shell, and silk fibroin/VEGF as the core materials. **Results:** The results observed that the core/shell nanofibers interact to differentiate MSCs into smooth muscle cells by the expression of vascular smooth muscle cell (VSMC) contractile proteins α-actinin, myosin and F-actin. **Conclusion:** The functionalized polycaprolactone/gelatin/silk fibroin/VEGF (250 ng) core/shell nanofibers were fabricated for the controlled release of VEGF in a persistent manner for the differentiation of MSCs into smooth muscle cells for vascular tissue engineering.

First draft submitted: 2 August 2018; Accepted for publication: 5 November 2018; Published online: 10 December 2018

Keywords: α-actinin • core/shell nanofibers • gelatin • mesenchymal stem cells • myosin • polymers • silk fibroin • smooth muscle cells • vascular tissue engineering • VEGF

Atherosclerosis and coronary arterial restenosis are common cardiovascular diseases causing medical problems globally. They require coronary artery bypass surgery where a blood vessel from the patient is explanted (autograft) in the place of a diseased blood vessel to lead a normal blood flow around the obstructed area and recondition the flow of blood to heart. Saphenous vein and native blood vessels (internal thoracic artery) are used to explant frequently in last 12 years for coronary artery bypass or peripheral bypass surgery due to characteristic difference between arteries and veins [1–3]. Synthetic vascular grafts are generally used for bypass surgery but it has several limitations such as infection, weak patency in the case of small diameter graft due to accumulation of thrombus formation on the lumen of the vessel and subsequently leads to failure of grafts [4,5]. The anatomy of blood vessels consists of intima, media and adventitia, within the media lies the smooth muscles cells (SMCs), which provide structural support to the vessels. Endothelial cells (ECs) cultured on vascular grafts have been proposed by many researchers; however, isolation of ECs from the patients causes the structure of the blood vessel to be sacrificed. Human mesenchymal stem cells (hMSCs) represent an alternative source of cells in the field of tissue engineering, which has many advantages over ECs [6–7]. The first tissue-engineered blood vessel was developed in 1986 by Weinberg and Bell using bovine ECs, SMCs and fibroblasts surrounded by collagen matrix [8]. Although, inappropriately, this study was not succeeded, later this work was placed as a key base for expressing the vital elements to fabricate tissue-engineered vascular graft (TEVG), and paved way in the field of vascular tissue engineering (VTE). Sultana *et al.* (2017) fabricated electrospun polycaprolactone (PCL)/chitosan mats with smooth and bead-free fiber structure to facilitate attachment and proliferation of ECs for the development of the inner layer of the blood vessel and

Future Medicine

SMCs for the formation of outer layer of artificial vascular grafts [9]. The fundamental elements of tissue engineering are cells and scaffolds interaction for the secretion of extracellular matrix (ECM) to the formation of new tissues [10]. The biocomposite scaffolds thus developed to address the limitations such as inflammation, toxicity and recognition due to the occurrence of scaffolds degradation [11–13]. Uchida *et al.* (2008) developed autologous TEVG, which should not be recognized as foreign object, attuned immunologically and free from thrombus formation for the regeneration of diseased blood vessels [14]. MSCs are one of the well-known sources in tissue engineering and have remarkable functions in the field of regenerative medicine [15]. They have an ability to form abundant types of tissues and organs, and can differentiate into multiple cell lineages [16,17]. MSCs cannot produce VEGF but VEGF can induce the cells upon differentiation into vascular cell types by the expression of PDGF receptors, which is a smooth muscle cell phenotypic modulator and angiogenic growth factor [18,19].

Electrospun nonwoven nanofibrous scaffolds are fabricated to mimic the function of native extracellular matrix (ECM) in vascular tissue engineering [20–22]. Core/shell is a versatile technique that has interesting functionalities over encapsulation of the active component, such as growth factors and drugs in core of fibers are beneficial in the field of drug delivery and regenerative medicine [23,24]. PCL is the US FDA-approved synthetic polymer known for its biodegradability, biocompatibility, permeability and good mechanical property [25–28]. Gelatin (GEL) is derived from natural component collagen, possesses same properties as collagen providing native environment for VSMCs; PCL is blended with GEL to obtain good mechanical stability for vascular grafts [29,30]. Fukunishi *et al.* (2016) demonstrated the vascular SMCs maturation in an electrospun biodegradable PCL/chitosan TEVG in the sheep model for engineering vascular grafts [31]. Silk fibroin (SF) is a fibrous protein, which tends to form a connective tissue microstructure and has enormous properties in the field of biomedical such as biocompatibility, immune response, permeability of water and oxygen for cell growth and chemical stability to mimic the *in vivo* environment of ECM [32,33]. *In vitro* study proved that SF nanofibers support VTE by maintaining vascular cell phenotype and backing the cell viability [34,35]. The present study is to develop functionalized core/shell nanofibers to mimic native ECM for the differentiation of MSCs into SMCs for VTE, which could be developed into vascular grafts beneficial for clinical perspective for bypass surgery in regenerative medicine.

Materials & methods

Materials

Polycaprolactone (MW 80,000), GEL from porcine skin type A (GEL strength ~300 g Bloom), 1,1,1,3,3,3-hexafluoro-2-propanol and 2,2,2-Trifluoroethanol were purchased from Sigma–Aldrich, Singapore; recombinant human VEGF was purchased from Abcam (Singapore); Alexa Fluor 488 was purchased from Invitrogen, Singapore; α-actinin and smooth muscle myosin were purchased from Abcam (Cambridge, MA, USA); Dulbecco's modified Eagle's medium, fetal bovine serum, antibiotic–antimycotic solutions, trypsin–EDTA were purchased from Sigma–Aldrich, Singapore; Alexa Fluor 647-Phalloidin was purchased from Molecular Probes by Life Technologies, Singapore; and CellTracker CMFDA green was purchased from Promega, Singapore. SF powders were obtained from Zhang Peng International Trading, Singapore; hMSCs from Lonza, Singapore.

Coaxial electrospinning for core/shell nanofibers

Coaxial electrospinning solutions of PCL/GEL were prepared separately at a 3:6 weight ratio, stirred overnight at room temperature and separately prepared solutions were added together to obtain uniform solutions. PCL/GEL/SF/VEGF core/shell nanofibers were prepared by dissolving 10% SF with VEGF (150 and 250 ng) as core and PCL/GEL solution loaded as shell, which has weight percentage of 3 and 6% in 1,1,1,3,3,3-hexafluoro-2-propanol. This process was performed using a 3 ml standard syringe, blunted 24-gauge needle, with the solution flow rate maintained as core 0.75 ml/h and shell 1.5 ml/h, controlled using syringe pump (KDS 100, KD Scientific, MA, USA). The distance between the needle tip and collector was set at 12–16 cm. A high voltage electric field of 9–13 kV (DC high voltage power supply from Gamma High Voltage Research, FL, USA) was applied to fabricate core/shell fibers from spinneret then collected on 15 mm coverslips placed over a grounded aluminium foil collector. Subsequently, the fabricated core/shell nanofibers were vacuum dried to remove the residual solvents present in the nanofibrous scaffolds. Quantitative analysis of VEGF release profile was performed in series of time period (day 1–15) by the ELISA-Quantikine human VEGF kit by the instructions of manufacturer protocol (R&D Systems, Novus Biologicals, Tocris Bioscience, MN, USA).

Characterization of the core/shell nanofibers

Core/shell nanofibrous scaffolds were observed under Field emission scanning electron microscope (FE-SEM, FEI-QUANTA 200F) for their surface morphology at 10 kV, followed by gold coating (JEOL JFC-1200 fine coater, JEOL, Tokyo, Japan). Random fibers were selected from every scaffold as n = 25 for measuring fibers diameter from the images of SEM. PCL/GEL/SF/VEGF core/shell nanofibers were observed by Transmission electron microscopy (TEM), using a JEOL 2100 microscope at an accelerating voltage of 200 kV. The width of fibers mentioned as diameter was calculated in average diameter alongside standard deviation by image analysis software (ImageJ, NIH, MD, USA). Core/shell fibers were examined using Fourier transform infrared (FTIR) spectroscopic analysis on Avatar 380, (Thermo Nicolet, MA, USA) for their functional group around the range of 500–4000 cm^{-1} at a resolution of 1 cm^{-1} and hydrophilic behavior of the nanofibers were analyzed under water contact angle, using VCA Optima surface analysis system (AST products, MA, USA).

Culturing of hMSCs

MSCs were cultured in 75 cm^2 flask with DMEM alongside 10% fetal bovine serum and 1% antibiotic–antimycotic solutions and incubated at 37°C on a humidified environment surrounding 5% CO_2 for 5 days and every alternative day changed the complete medium. The cells grown in 75 cm^2 cell culture flasks were trypsinized by adding 1 ml of 0.25% trypsin containing 0.1% EDTA to lift cells in the flasks and then centrifuged for collecting cells. Trypan blue assay used to count the cells in hemocytometer and then cells were seeded onto the scaffolds at a seeding density of 10,000 cells per well. Cells seeded on Tissue culture plate (TCP) were used as a control to monitor the morphology, growth and differentiation of cells.

Cell proliferation

Cell proliferation was analyzed in PCL/GEL, PCL/GEL/SF, PCL/GEL/SF/VEGF (150 & 250 ng) and TCP core/shell nanofibrous scaffolds by MTS assay (CellTiter 96 AQueous Assay) for a period of 5, 10 and 15 days. The core/shell nanofibers were sterilized by treating UV light for 3 h. The sterilized fibers were placed in 24-well plate with stainless steel rings, in order to avoid lifting of fibers upwards followed by washing in phosphate-buffered saline (PBS) to remove the residual solvents consequently; the fibers were then kept overnight in DMEM before seeding cells. After culturing cells on nanofibers (10,000 cells/well), the cells were washed with PBS to remove the dead and unattached cells subsequently then MTS reagent (1:4) in serum-free media was added followed by incubation for 3 h in the incubator, which maintains 37°C in 5% CO_2. After addition of MTS reagent, formation of purple formazan crystals was observed, which results in reduction of tetrazolium salt by dehydrogenase enzymes secreted by mitochondria of metabolically active cells. The dye obtained thereby was transferred to 96-well plate to measure at 490 nm, using a spectrophotometric plate reader (FLUOstar OPTIMA, BMG Lab Technologies, Ortenberg, Germany). Intensity of formazan dye found is directly proportional to the number of cells.

Cell morphology

FE-SEM (FEI-QUANTA 200F) was used to determine the morphology of cells for the day 10. Cell culture medium was removed from the cells scaffolds and washed with PBS two times. Subsequently, the cells were fixed with 3% glutaraldehyde in cold PBS for 3 h. The scaffolds were washed with distilled water for 15 min and dehydrated with a series of ethanol gradients starting from 30, 50, 75, 90 to 100% (v/v) followed by treating the samples with hexamethyldisilazane (HMDS) solution and allowed to air-dry at room temperature in the fume hood. The samples were then removed and coated with gold and analyzed in FE-SEM.

Expression of 5-chloromethyl-fluorescein-diacetate dye

Cultured cells were stained with green fluorescent dye 5-chloromethyl-fluorescein-diacetate (CMFDA) to track the live cells. CMFDA dye passes the live cell membrane and transforms into the cell membrane as an impermanent reaction, which results in monitoring live cells. Before adding the fluorescent dye to scaffold-containing cells, medium should be removed by the addition of CMFDA dye with serum-free DMEM at a concentration of 20 µl (25 µM)/180 µl and incubated at 37°C for 3 h in an incubator. Subsequently, the CMFDA dye should be removed and replaced by the complete DMEM to incubate at 37°C for 24 h. After a day, the medium was removed from the scaffold-containing cells then washed with PBS to remove the dye and dead cells. Serum-free media of 200 µl added to the scaffolds, which was observed under an inverted Leica DM IRB laser-scanning microscope, Wetzlar, Germany (Leica DC 300F) at 488 nm.

Expression of collagen

Sirius red staining (0.1%) was performed to determine the secretion of collagen in the cell matrix qualitatively. Initially, the cells were washed with PBS, fixed in 10% formaldehyde, and then 200 µl of hematoxylin was added to incubate for 1 h. Followed by washing with deionized water thrice, the cell scaffolds were stained with Sirius red for 1 h and subsequently washed with mild acidified water to remove unbounded dye to capture images. Finally, the expression of collagen stained red on a yellow background in the nanofibrous scaffolds was viewed under Leica DM IRB microscope.

Immunofluorescence analysis by Confocal laser scanning microscope (CLSM)

Expression of α-actinin & myosin

Expression of contractile-specific marker protein was observed on the differentiated cells cultured on the nanofibrous scaffolds. For CLSM analysis, on day 15, the cells were fixed with 100% ice-cold methanol before processing. The samples were then washed with PBS once for 15 min and incubated in 0.5% Triton-X solution (Sigma–Aldrich, Singapore) in order to make the cell membrane permeable; the nonspecific binding sites were blocked by adding 3% bovine serum albumin (BSA), for 1 h. Subsequently, the primary antibodies α-actinin and myosin heavy chain were added in a dilution of 1:100 for 90 min at room temperature. Later the samples were washed with PBS for 15 min to remove the excess unbound primary antibodies. This was followed by incubation with Alexa Fluor 488 secondary antibodies in a dilution of 1:250 for 60 min at room temperature. The samples were washed with PBS for 15 min and stained with diamidino-2-phenylindole dihydrochloride (DAPI – 1:2000 dilution) for 30 min at room temperature. The cells were again washed with PBS, then the samples were removed and mounted over glass slides using Vectashield mounting medium (Sigma–Aldrich, Singapore) and observed under Olympus FV1000 CLSM (Olympus Singapore Pte Ltd, Singapore).

F-actin

Cells seeded on core/shell nanofibrous scaffolds were stained for the expression of F-actin on day 15 of cell culture. Cells fixed with ice cold methanol, were washed with PBS and incubated with 0.5% Triton-X100 to make the cell membrane permeable for 5 min. Upon addition of 3% BSA, which blocks the nonspecific sites, the samples were added with Alexa Fluor 647-Phalloidin and incubated for 90 min at room temperature in the dilution of 1:100. The samples were incubated with DAPI dilution of 1:2000 for 30 min followed by washing in PBS to remove the excess staining. The samples were mounted in glass slide with the help of mounting medium and examined under Olympus FV1000-CLSM.

Statistical analysis

Experimental data were presented as mean standard deviation (n = 6). Statistical differences between the groups were analyzed using one-way analysis of variance using Tukey's post-hoc analysis. Statistical significance was represented as $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

Results

Characterization of core/shell nanofibers

Core/shell nanofibers fabricated to mimic the native ECM for the differentiation of MSCs into VSMCs for the regeneration of vascular tissues.Figure 1 shows the morphology of PCL/GEL, PCL/GEL/SF, PCL/GEL/SF/VEGF (150 ng) and PCL/GEL/SF/VEGF (250 ng) core/shell nanofibers. The fiber diameter obtained in the range of 753 ± 44 for PCL/GEL, 656 ± 52 for PCL/GEL/SF, 478 ± 70 for PCL/GEL/SF/VEGF (150 ng) and 454 ± 68 nm for PCL/GEL/SF/VEGF (250 ng). The TEM image is showing the double layer of core and shell separately for PCL/GEL/SF/VEGF nanofibers (Figure 1F). The water contact angle of nanofibrous scaffolds observed PCL/GEL approximately 56.6 ± 7.1°, PCL/GEL/SF ~ 36.2 ± 4.3°, PCL/GEL/SF/VEGF (150 ng) ~46.5 ± 5.1° and PCL/GEL/SF/VEGF (250 ng) ~44.7 ± 4.7° (Table 1).Figure 2 shows PCL/GEL, PCL/GEL/SF, PCL/GEL/SF/VEGF (150 ng) and PCL/GEL/SF/VEGF (250 ng) of coaxial electrospinning nanofibers have tensile strengths of approximately 3.0, 3.3, 2.8 and 2.6 MPa (Megapascal). FTIR results show the characteristic peaks of PCL, GEL, SF and VEGF (Figure 3). The peaks at 3295 and 1636 cm^{-1} were observed as N-H stretching and C = O amide bond stretching for GEL, respectively. C = O ester stretching vibration as a prominent peak at 1725 cm^{-1} (saturated aldehyde) was observed in all nanofibrous scaffolds, which confirms the presence of PCL. FTIR spectrum shows the important functional groups such as amine 1, 2 and 3 in infrared (IR)

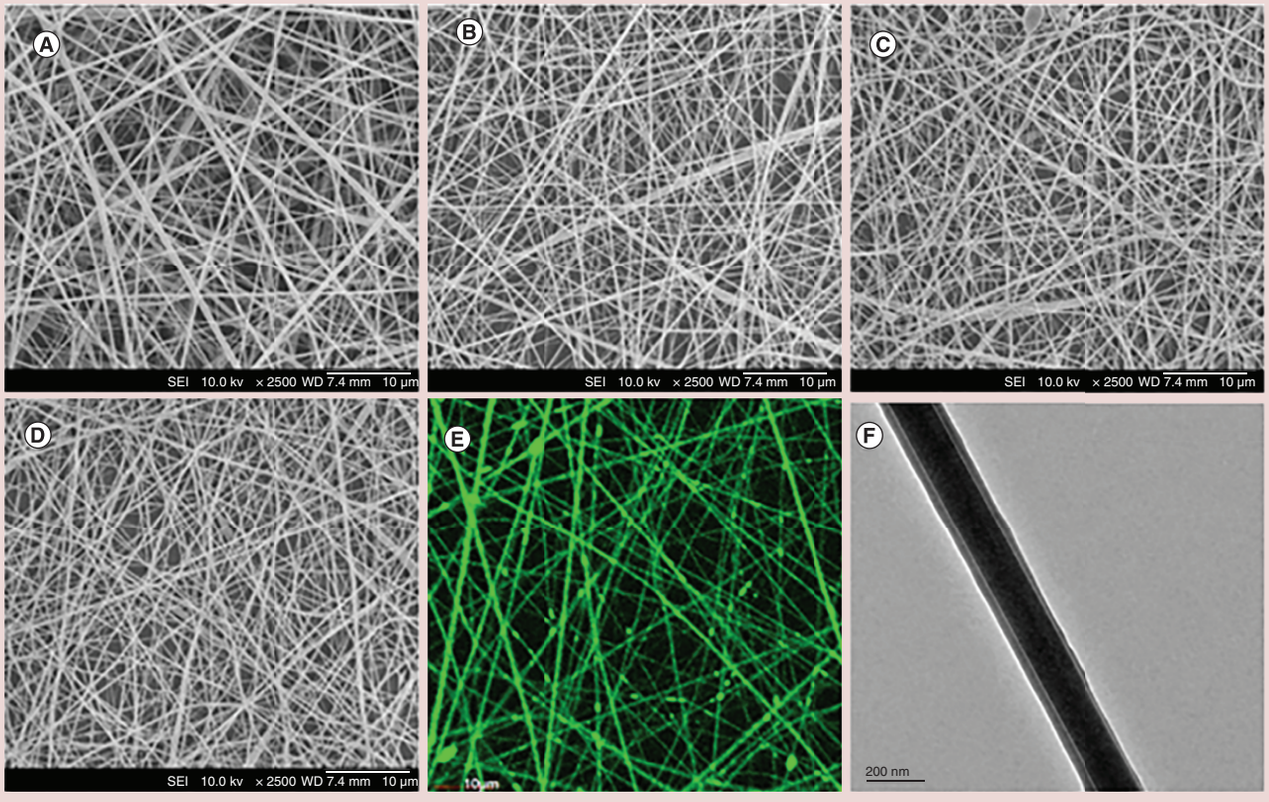


Figure 1. Scanning electron microscopy images of biocomposite functionalized core/shell nanofibers. (A) PCL/GEL, **(B)** PCL/GEL/SF, **(C)** PCL/GEL/SF/VEGF (150 ng), **(D)** PCL/GEL/SF/VEGF (250 ng) and **(E)** fluorescein isothiocyanate fluorescent image. **(F)** Transmission electron microscopy image of core/shell nanofibers.
GEL: Gelatin; PCL: Polycaprolactone; SF: Silk fibroin.

Table 1. Characterization of functionalized core/shell nanofibers for contact angle, fiber diameter, tensile stress and Young's modulus.

Core/shell nanofibers	Fiber diameter (nm)	Water contact angle (°)	Tensile stress (MPa)	Young's modulus (MPa)
PCL/GEL	753 ± 44	56.6 ± 7.1	3.0	30.2
PCL/GEL/SF	656 ± 52	36.2 ± 4.3	3.3	33.7
PCL/GEL/SF/VEGF 150 ng	478 ± 70	46.5 ± 5.1	2.8	34.9
PCL/GEL/SF/VEGF 250 ng	454 ± 68	44.7 ± 4.7	2.6	32.4

GEL: Gelatin; MPa: Megapascal; PCL: Polycaprolactone; SF: Silk fibroin.

peaks as bending vibration 1636 and 1537 (NH_2), and C-N stretching 1240 cm^{-1} respectively, in scaffolds loaded with SF. The cumulative VEGF release profile was detected initially very low on day 2, later growth factor release was significantly increased after day 10 for initiating the cell differentiation leads to SMCs (Supplementary Figure 1).

Biological activity of cells

The proliferation of cells in PCL/GEL, PCL/GEL/SF, PCL/GEL/SF/VEGF (150 ng) and PCL/GEL/SF/VEGF (250 ng) nanofibers was monitored for a time period of days 5, 10 and 15 to prove the scaffolds that were not cytotoxic for culturing of cells (Figure 4). Initially on day 5, PCL/GEL/SF/VEGF (150 ng) and PCL/GEL/SF/VEGF (250 ng) were showing normal cell proliferation rate similar to that of PCL/GEL/SF nanofibers. Subsequently, on

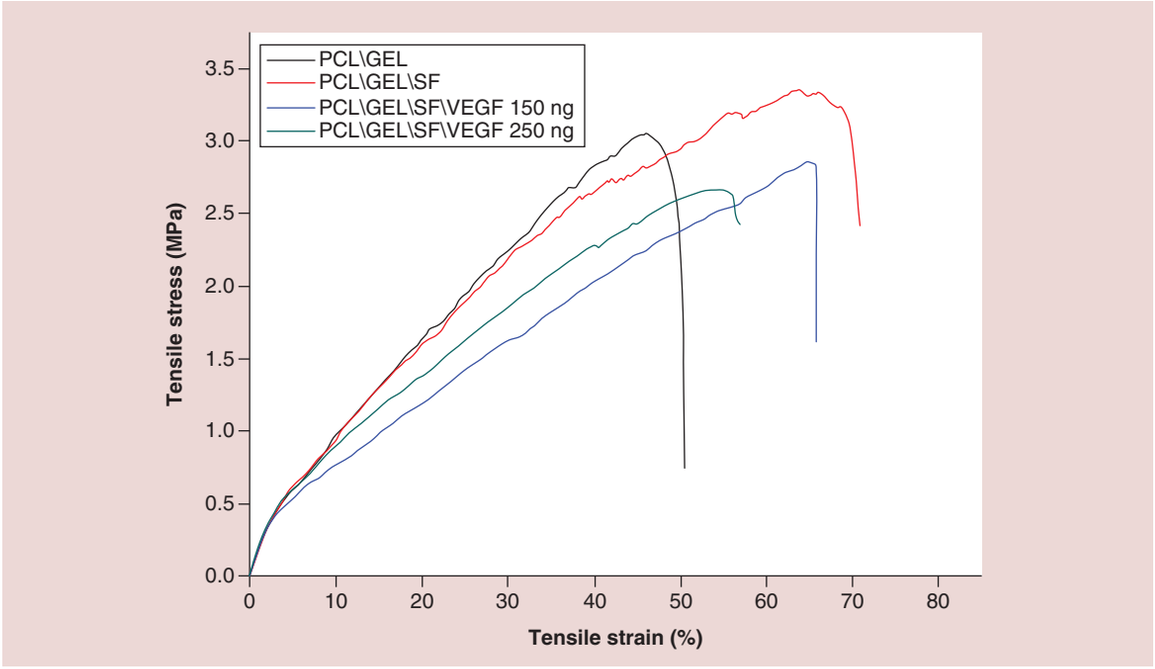


Figure 2. Tensile properties of PCL/GEL, PCL/GEL/SF, PCL/GEL/SF/VEGF (150 ng) and PCL/GEL/SF/VEGF (250 ng) functionalized core/shell nanofibrous scaffolds.
GEL: Gelatin; PCL: Polycaprolactone; SF: Silk fibroin.

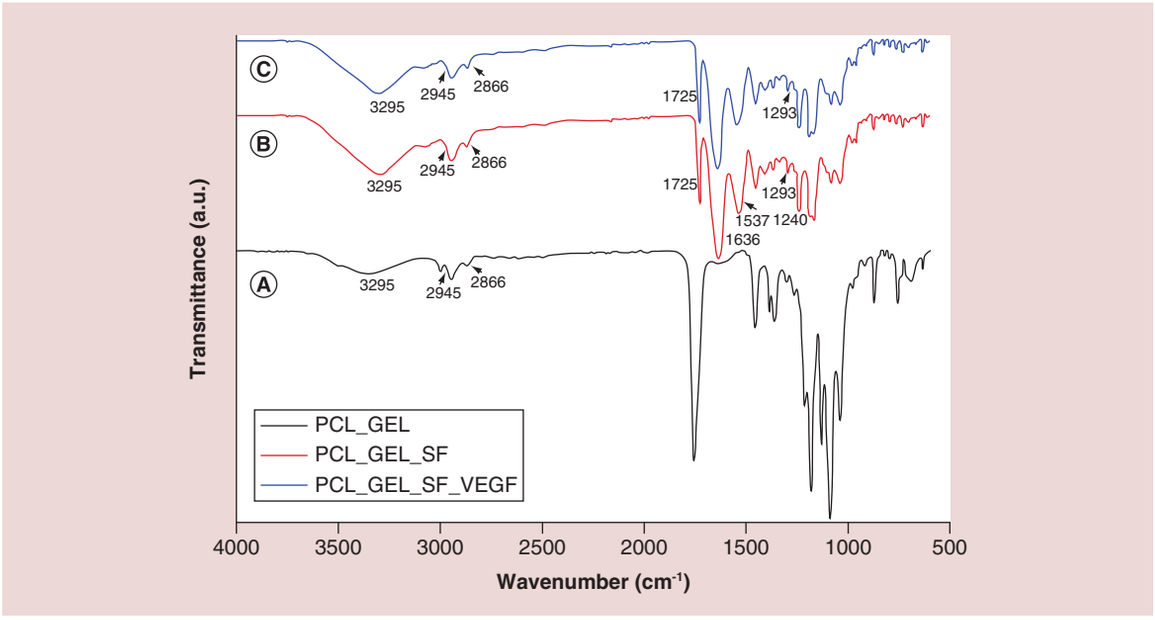


Figure 3. Fourier transform infrared spectra of functionalized biocomposite core/shell nanofibrous scaffolds. (A) PCL/GEL, **(B)** PCL/GEL/SF and **(C)** PCL/GEL/SF/VEGF (250 ng).
GEL: Gelatin; PCL: Polycaprolactone; SF: Silk fibroin.

day 10 decreased cell proliferation was observed in PCL/GEL/SF/VEGF 150 ng (5%) and PCL/GEL/SF/VEGF 250 ng (12.6%) compared with PCL/GEL/SF scaffolds and also on day 15, scaffolds were loaded with VEGF 150 ng (14.35%) and 250 ng (16%). Fluorescent imaging was captured on the cells seeded nanofibrous scaffolds under the influence of CMFDA dye. CMFDA fluorescence dye penetrates through cell membrane of viable cells, where it is

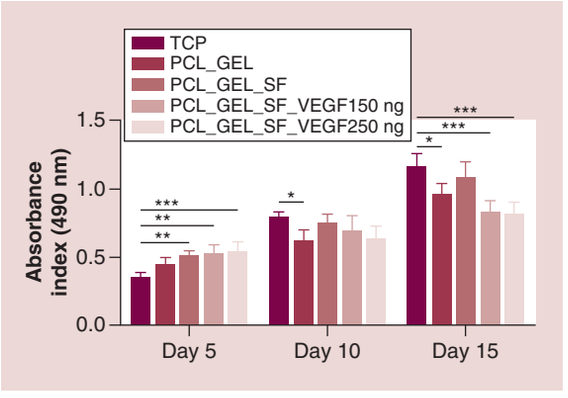


Figure 4. Proliferation of mesenchymal stem cells on tissue culture plate, PCL/GEL, PCL/GEL/SF, PCL/GEL/SF/VEGF (150 ng), and PCL/GEL/SF/VEGF (250 ng) functionalized biocomposite core/shell nanofibrous scaffolds. Data are presented as mean ± standard deviation with n = 6 (*p ≤ 0.05, **p < 0.01, ***p < 0.001).
GEL: Gelatin; PCL: Polycaprolactone; SF: Silk fibroin.

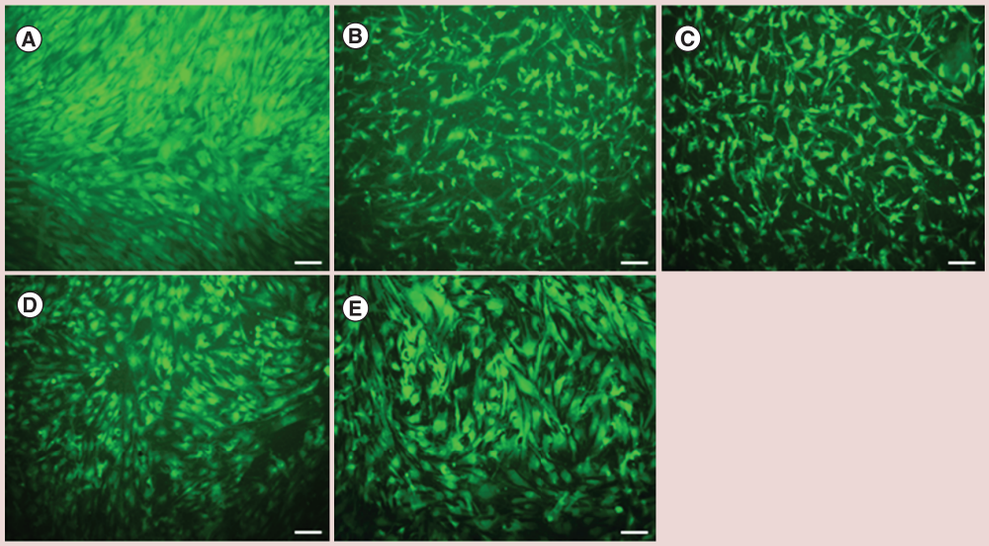


Figure 5. The 5-chloromethyl-fluorescein-diacetate dye for live cell imaging on functionalized biocomposite core/shell nanofibrous scaffolds on day 10. (A) Tissue culture plate, **(B)** PCL/GEL, **(C)** PCL/GEL/SF, **(D)** PCL/GEL/SF/VEGF (150 ng), and **(E)** PCL/GEL/SF/VEGF (250 ng), scale bar = 50 μm.
GEL: Gelatin; PCL: Polycaprolactone; SF: Silk fibroin.

changed into cells impermeant and exhibits a fluorescence acting upon cystolic esterase in cells within 60 min. The cells tagged with chloromethyl derivative of CMFDA respond with polypeptide that carries thiols, which leads to bright fluorescence expressed in viable cells. Figure 5 displays the CMFDA dye expression in hMSCs cultured on TCP and all other core/shell nanofibrous scaffolds. Sirius red staining was used to analyze the secretion of collagen in cells cultured on core/shell nanofibers, higher level of collagen secretion was observed in PCL/GEL/SF/VEGF 250 ng nanofibers compared with all other nanofibrous scaffolds (Figure 6). Generally, morphology of the cells in response to biomolecules present in the nanofibrous scaffolds, the cells–scaffold interaction depends on cell adhesion, proliferation, migration and differentiation leads to develop vascular grafts (Figure 7). The results showed that vascular proteins such as α-actinin, myosin and F-actin are expressed in differentiated MSCs cultured on PCL/GEL, PCL/GEL/SF, PCL/GEL/SF/VEGF (150 ng) and PCL/GEL/SF/VEGF (250 ng) core/shell nanofibrous scaffolds for the regeneration of vascular tissues (Figure 8).

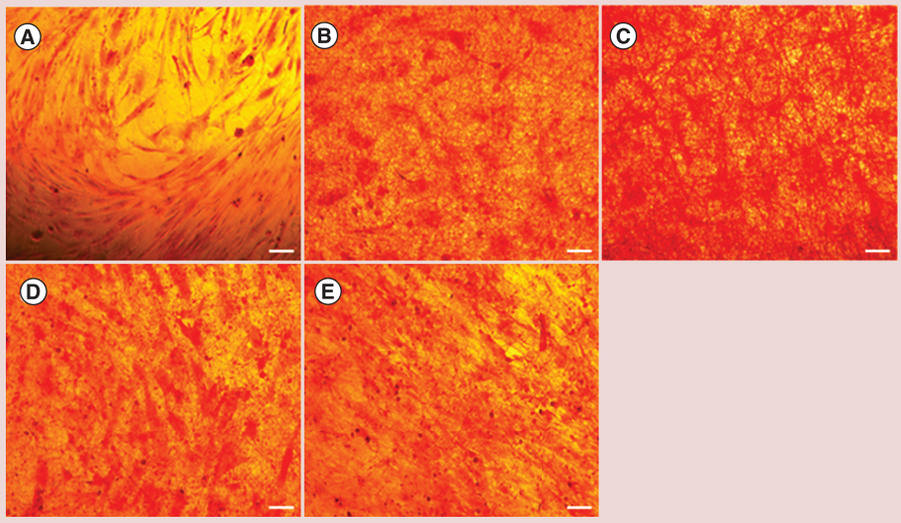


Figure 6. Sirius red staining for collagen expression on day 10. (A) Tissue culture plate **(B)** PCL/GEL, **(C)** PCL/GEL/SF, **(D)** PCL/GEL/SF/VEGF (150 ng), and **(E)** PCL/GEL/SF/VEGF (250 ng) core/shell nanofibers, scale bar = 50 μm.
GEL: Gelatin PCL: Polycaprolactone; SF: Silk fibroin.

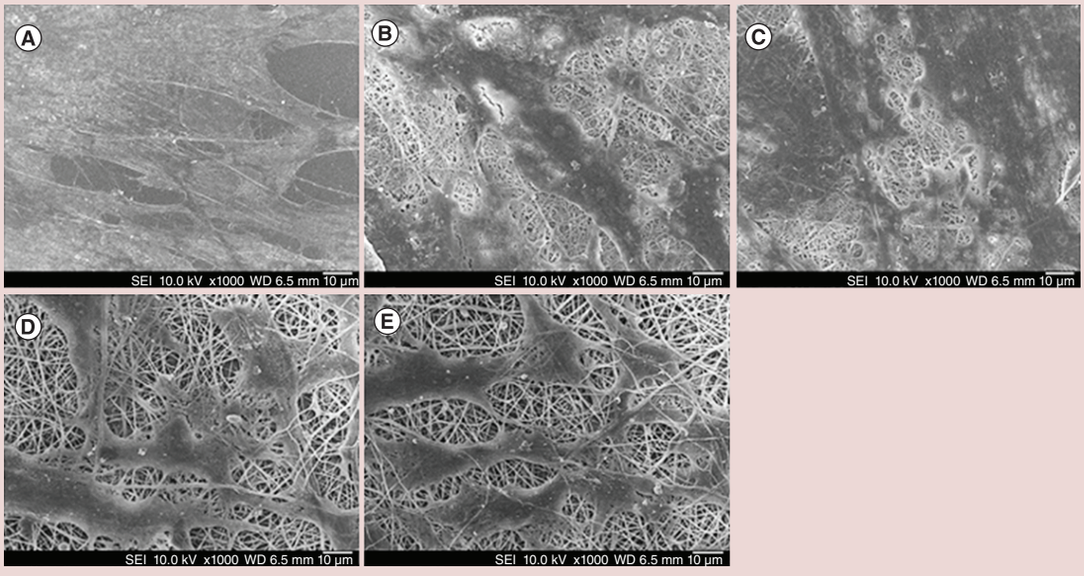


Figure 7. Morphology of the mesenchymal stem cells cultured on the functionalized biocomposite core/shell nanofibrous scaffolds on day 10. (A) TCP, **(B)** PCL/GEL, **(C)** PCL/GEL/SF, **(D)** PCL/GEL/SF/VEGF (150 ng), and **(E)** PCL/GEL/SF/VEGF (250 ng).
GEL: Gelatin; PCL: Polycaprolactone; SF: Silk fibroin.

Discussion

Characterization of core/shell nanofibers

Core/shell nanofibers fabricated in nanoscale dimension by synthetic and natural polymers, which is biodegradable in nature to mimic the natural ECM with high porosity, well interconnected pores and large surface area for better ingrowth of cells for tissue engineering applications. The fiber diameter of PCL/GEL nanofibers was obtained 753 ± 44 nm, which decreases in the presence of SF (PCL/GEL/SF ~ 656 ± 52 nm), further decreased upon incorporation of VEGF (PCL/GEL/SF/VEGF [150 ng] ~ 478 ± 70 nm; PCL/GEL/SF/VEGF [250 ng] ~

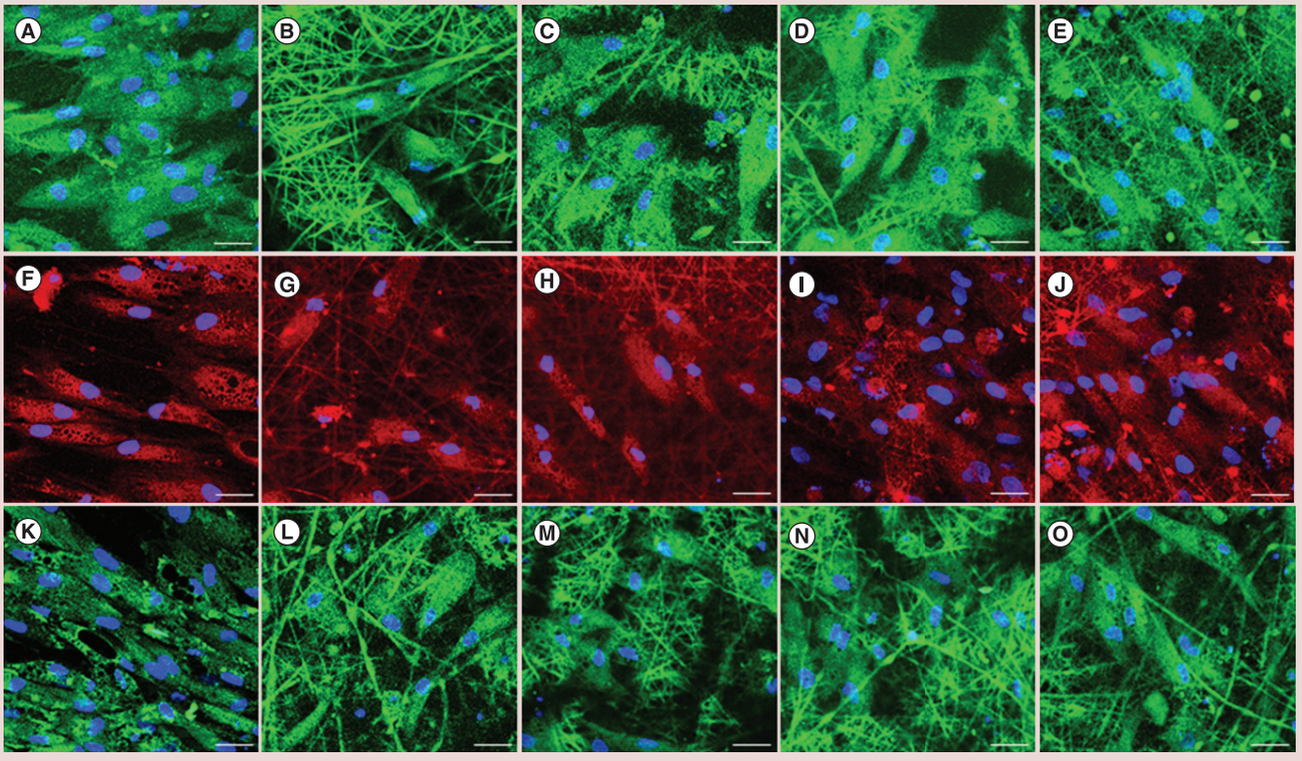


Figure 8. Immunofluorescence analysis of human mesenchymal stem cells. (A–E) Expression of VSMCs-specific marker protein α-actinin (green) on day 15, **(F, G, H, I, J)** smooth muscle myosin (red), and **(K, L, M, N, O)** phalloidin for expression of F-actin on tissue culture plate **(A, F, K)**, PCL/GEL **(B, G, L)**, PCL/GEL/SF **(C, H, M)**, PCL/GEL/SF/VEGF (150 ng) **(D, I, N)**, PCL/GEL/SF/VEGF (250 ng) **(E, J, O)**, nucleus stained with DAPI (blue), scale bar = 20 μm.
GEL: Gelatin; PCL: Polycaprolactone; VSMC: Vascular smooth muscle cell; SF: Silk fibroin.

454 ± 68 nm; Figure 1). This decrement could be assigned to increase in conductivity of the solution with SF and VEGF. The decrease in fiber diameter containing VEGF was also proved by Tian *et al.* [36]. Natural and synthetic polymers were blended for the surface modification of core/shell nanofibers, which influence cell adhesion and proliferation by the impact of functional groups such as hydroxyl, amine and carboxylic group. The hydrophilicities measured on the nanofibrous scaffolds were PCL/GEL approximately 56.6 ± 7.1°, PCL/GEL/SF∼36.2 ± 4.3°, PCL/GEL/SF/VEGF (150 ng)∼46.5 ± 5.1° and PCL/GEL/SF/VEGF (250 ng) ∼44.7 ± 4.7° (Table 1). Generally observed that all scaffolds have contact angle <90°, which is favorable for cell adhesion and proliferation in vascular tissue engineering.

Core/shell formation evaluation, a control experiment was done using FITC–BSA system as core and PCL/GEL as shell; Figure 1E shows emission of fluorescent light in the presence of FITC, thereby conforming the core/shell formation. The control experiment clearly demonstrated the distribution of growth factor (protein) in the core of PCL/GEL/SF/VEGF nanofibers. PCL/GEL, PCL/GEL/SF, PCL/GEL/SF/VEGF (150 ng) and PCL/GEL/SF/VEGF (250 ng) of coaxial electrospinning nanofibers have tensile strengths approximately 3.0, 3.3, 2.8 and 2.6 MPa (Figure 2 & Table 1). Tensile strength of the nanofiber scaffolds loaded with silk fibroin has increased in comparison with scaffolds without PCL/GEL fibers; the increase in mechanical property is due to high-density ratio of silk fibroin and β sheet, which help to form a polymer network by crosslinking fibroins together [37]. Mrówczyński *et al.* (2014) proved that the biodegradable, electrospun PCL grafts showed good surgical and mechanical properties, endothelialization, no aneurysm formation, and similar short-term patency compared with poly(tetrafluoroethylene) (ePTFE) grafts [38]. Results observed that VEGF added into PCL/GEL/SF decreased mechanical strength of PCL/GEL/SF/VEGF (150 ng) to 2.8 MPa, PCL/GEL/SF/VEGF (250 ng) to 2.6 MPa compared with PCL/GEL and PCL/GEL/SF nanofibers. Kai *et al.* (2015) demonstrated similar results of lower

mechanical property in nanofibers by incorporation of VEGF [39]. Human coronary artery has tensile stress of 1.4 MPa [40]. FTIR spectrum shows in Figure 3, the saturated aldehyde peak at 1725 cm^{-1} and specific functional groups such as amine 1, 2 and 3 in IR peaks as bending vibration 1636 and 1537 (NH_2), and C-N stretching 1240 cm^{-1} respectively, in PCL fibrous scaffolds loaded with SF [41,42]. VEGF and SF proteins share same surface functional moieties such as amine (NH) and carboxylic group (COOH), and its characteristic peaks may have diffused with that of SF. General protein band displays at 1636 cm^{-1} (amine 1) and 1537 cm^{-1} (amine 2) conforming the stretching vibration of C = O, C-N to bending vibration of N-H in functionalized core/shell nanofibers.

Biological characterization *in vitro*

Cell proliferation

Proliferation of cells was monitored for a series of time periods 5, 10 and 15 days (Figure 4). Initially on day 5, PCL/GEL/SF/VEGF (150 ng) and PCL/GEL/SF/VEGF (250 ng) were showing normal cell proliferation rate similar to that of PCL/GEL/SF nanofibers. The cell proliferation was decreased in PCL/GEL/SF/VEGF 150 ng (5%) and PCL/GEL/SF/VEGF 250 ng (12.6%) compared with PCL/GEL/SF core/shell nanofibers on day 10; and also on day 15, core/shell nanofibers were loaded with VEGF 150 ng (14.35%) and 250 ng (16%) %). Ravichandran *et al.* (2013) reported that the rate of proliferation of cells begins to decrease while differentiation of stem cells due to withdrawal of cell cycle and become terminally differentiated cells [43]. The results also obtained for VEGF-loaded nanofibrous scaffolds on day 15 show decrease in proliferation due to the differentiation of hMSCs to VSMCs. PCL/GEL/SF nanofibers display higher proliferation compared with PCL/GEL, which is due to the influence of SF, the natural compound helps cells to attach and grow better by supporting cell viability and maintaining normal cell phenotype [44,45].

Expression of 5-chloromethyl-fluorescein-diacetate

CMFDA is cell-tracker dye, provides sharp fluorescence that differentiate among cells and nanofibers, which helps to observe the size, shape and morphology of viable cells *in vitro*. Figure 5 displays the CMFDA dye expression in hMSCs cultured on TCP and all other core/shell-functionalized nanofibrous scaffolds. A clear difference in cell morphology was observed in scaffolds with and without VEGF. VEGF loaded core/shell fibers showing well-aligned elongated spindle shaped cells with notable cell to cell interaction within the cells scaffolds. The PCL/GEL and PCL/GEL/SF, without VEGF display irregular spherical shaped cells with notable cell to cell interactions due to the presence of biopolymers. Subsequently, the cells in PCL/GEL/SF/VEGF 250 ng (Figure 5E) showed more MSCs differentiated into elongated spindle-shaped SMCs-like morphology with good interconnection of cells and well-supported growth along the surface of core/shell fibrous scaffolds compared with all other nanofibrous scaffolds.

Secretion of collagen

Picro-Sirius red staining is the marker for qualitative analysis of collagen to confirm the secretion of ECM in cells cultured on the core/shell nanofibrous scaffolds. Sulfonic acid group of anionic dye from Picro-Sirius red interacts with the basic groups of collagen, which tend to show the stained red color. Collagen is a specific protein, highly responsible for the strength and elasticity of any skin coverings and is one of the major ECM. Collagen secretion was notably observed in all samples after day 10. Subsequently, Figure 6E (PCL/GEL/SF/VEGF 250 ng) observed higher levels of collagen secretion compared with all other nanofibrous scaffolds. It becomes apparent that the cells secretion of collagen in biocomposite nanofibrous scaffolds are likely to initiate blood vessel tissue regeneration.

Morphology of cells in core/shell nanofibers

Figure 7 shows the interaction of cells-scaffolds progression of appropriate biological responses such as adhesion, cell morphology, growth, migration and differentiation of cells by core/shell nanofibers fabricated with biocompatible natural polymers and growth factors. Hybridization of PCL and GEL provides a better rough surface for cell binding site and good mechanical property for nanofibers. PCL lacks the hydrophilic nature, which is significant for cell communication; GEL does not exhibit good mechanical properties. VEGF-loaded PCL/GEL/SF nanofibers were chosen to observe the VSMCs differentiation from hMSCs that further leads to the formation of vascular grafts. PCL/GEL and PCL/GEL/SF shows better adhesion and growth of cells compared with TCP due to more number of protein binding sites provided by SF and GEL on day 10. PCL/GEL and PCL/GEL/SF observed that the cells penetrated into nanofibrous scaffolds through enhanced migration and cellular communication; thereby proving the

biological advantages of nanofibers. Cells in PCL/GEL/SF/VEGF (150 ng) and PCL/GEL/SF/VEGF (250 ng) nanofibers showed that cells were well aligned, interconnected with spindle-shaped cells and elongated VSMCs phenotypic morphology observed in the differentiated cells. Density of cells in PCL/GEL/SF/VEGF (150 ng) and PCL/GEL/SF/VEGF (250 ng) was low compared with PCL/GEL and PCL/GEL/SF, which is due to the differentiation induced by bioactive VEGF incorporated with SF core/shell nanofibers.

VSMCs marker proteins

During the stage of vascular diseases, smooth muscle cells of the blood vessel lose their contractile proteins and increased their proliferation, migration and secretion of ECM proteins. VSMCs remain very particular to maintain the functions of contraction and help to control the blood flow and blood pressure. Contractile proteins of SMCs play a major role in the development of blood vessels, also regulate the diameter of blood vessel during blood flow. In order to witness the differentiation of hMSCs into VSMCs, the immunofluorescence-specific marker proteins are analyzed, which are significant for contractile properties of SMCs [46]. VSMCs express the cytoskeletal proteins, myosin and α-actinin, to maintain the cell phenotypes [47]. The observed results showed more or less similar to the differentiation of MSCs into the contraction and dilatation properties of SMCs as proved by Sultana *et al.* (2017) in vascular tissue engineering [9]. Figure 8 shows the expression of actinin and myosin filament on the core/shell nanofibers is obvious compared with TCP, which means immunofluorescent labeling shows greater amount of actinin and myosin expression in core/shell nanofibers. The results show that hMSCs cultured on PCL/GEL/SF/VEGF (150 ng) and PCL/GEL/SF/VEGF (250 ng) have experienced complete differentiation by controlled release of VEGF in core/shell nanofibers and it is evident from their morphology of VSMCs and expression of marker proteins. The expression of contractile proteins, α-actinin and myosin, was low in PCL/GEL and PCL/GEL/SF while in PCL/GEL/SF/VEGF (150 ng) and PCL/GE/SF/VEGF (250 ng) obtained higher levels of proteins for proving the differentiation of MSCs into vascular tissue engineering (Figure 8).

Phalloidin stains the filaments of actin, which is expressed in cells by binding of actin in the polymerized form. F-actin is one of the significant proteins for all cell types including VSMCs and also plays an important role in cell contraction that helps to bear the stress of blood flow by actin filaments. Zhou *et al.* (2016) demonstrated that the two-layered bioengineered vessels (PCL/GEL) exhibited biomechanical properties similar to normal human saphenous veins and also to differentiate the human adipose-derived stem cells differentiated into SMCs and ECs for bioengineering small-diameter blood vessels [48]. The observed results of PCL/GEL/SF/VEGF (150 ng) show higher level of F-actin expression than PCL/GEL, PCL/GEL/SF and TCP. Subsequently, in Figure 8J, PCL/GEL/SF/VEGF (250 ng) shows more expression of F-actin in comparison to all other core/shell nanofibrous scaffolds. The results proved that the PCL/GEL/SF/VEGF (250 ng) and 150 ng functionalized core/shell nanofibrous scaffolds induced the differentiation of MSCs into spindle-shaped VSMCs with increased expression of specific marker proteins, thereby evidencing the regeneration of vascular tissue in regenerative medicine.

Conclusion

Coaxial electrospinning process used to fabricate PCL/GEL, PCL/GEL/SF, PCL/GEL/SF/VEGF (150 ng) and PCL/GEL/SF/VEGF (250 ng) functionalized core/shell nanofibers. These core/shell nanofibers supported the cell growth in case of cell adhesion, migration and proliferation by inducing suitable environment alike native ECM for vascular tissue engineering. It was shown by contact angle measurements that the nanofibrous scaffolds containing VEGF exhibited significantly higher surface hydrophilicity compared with PCL/GEL and PCL/GEL/SF nanofibers. Core/shell nanofibrous scaffolds containing growth factor (VEGF) showed a prominent change in morphology and proliferation of cells by inducing the therapeutic angiogenesis by VEGF. The scaffolds with SF, which has a wide range of vascular tissue engineering potential as effective property over drug release and good mechanical property, incorporated with VEGF show low proliferation rate by means of differentiation of the hMSCs to VSMCs. These studies demonstrated that functionalized core/shell nanofibers loaded with VEGF have good potential for the regeneration of new tissue by its bioactivity. The bioactive PCL/GEL/SF/VEGF (250 ng) core/shell nanofibrous scaffolds fabricated by coaxial electrospinning based on silk fibroin make them efficient for controlled releasing VEGF in a persistent manner and widely favorable scaffolds in vascular tissue engineering.

Future perspective

Atherosclerosis and coronary arterial restenosis are common cardiovascular diseases causing medical problems globally, which demand coronary artery bypass surgery. The surgical bypass grafting mostly involves the use of autologous vessels, such as the saphenous vein and internal thoracic artery. Synthetic vascular graft is an alternative to the natural conduits, but it has several limitations such as infection, weak patency in the case of small diameter vascular graft due to an accumulation of thrombus in the lumen of the vessel and subsequently leads to the failure of grafts. The functionalized core/shell nanofibers supported cell growth, migration, proliferation and differentiation by inducing suitable environment alike native ECM for developing vascular grafts. The drawback using synthetic grafts is to resolve the problem, we developed functionalized core/shell nanofibers grafts play a role in the controlled delivery of growth factors to initiate the differentiation of mesenchymal stem cells into vascular smooth muscle cells to develop vascular grafts more beneficial for clinical perspective for bypass surgery in regenerative medicine.

Summary points

- Coaxial electrospinning technique used for the fabrication of core/shell nanofibers contained polycaprolactone/gelatin (PCL/GEL) as the shell, and silk fibroin (SF)/VEGF (150 & 250 ng) as the core materials.
- The core/shell fiber diameter increased in PCL/GEL nanofibers, which was decreased significantly in SF/VEGF nanofibers by increasing the conductivity of the solution.
- VEGF-loaded core/shell nanofibrous scaffolds observed decreased in cells proliferation compared with PCL/GEL/SF nanofibers due to the differentiation of human mesenchymal stem cells to VSMCs.
- Mesenchymal stem cells cultured on PCL/GEL/SF/VEGF (250 ng) showed differentiation of smooth muscles cells by the controlled release of VEGF in core/shell nanofibers and also proved by the expression of α-actinin, myosin and F-actin marker proteins for vascular tissue engineering.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/nnm-2018-0271

Financial & competing interests disclosure

This study was supported by the NUS-CNN, Department of Mechanical Engineering, Faculty of Engineering, National University of Singapore, Singapore and Faculty of Industrial Sciences & Technology, Universiti Malaysia Pahang, Malaysia. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

References

Papers of special note have been highlighted as: • of interest; •• of considerable interest

1. Rayatpisheh S, Heath DE, Shakouri A, Rujitanaroj P-O, Chew SY, Chan-Park MB. Combining cell sheet technology and electrospun scaffolding for engineered tubular, aligned, and contractile blood vessels. *Biomaterials* 35(9), 2713–2719 (2014).
2. Cleary MA, Geiger E, Grady C, Best C, Naito Y, Breuer C. Vascular tissue engineering: the next generation. *Trends Mol. Med.* 18(7), 394–404 (2012).
3. Sheppard MN. Complications following cardiac coronary artery bypass surgery. *Diagnostic Histopathol.* 18, 468–477 (2012).
4. Jia L, Prabhakaran MP, Qin X, Ramakrishna S. Stem cell differentiation on electrospun nanofibrous substrates for vascular tissue engineering. *Mater. Sci. Eng. C* 33(8), 4640–4650 (2013).

•• **Characterization of poly-L-lactide/collagen nanofibers and differentiating mesenchymal stem cells into endothelial cells for vascular regeneration.**

5. Liu Tsang V, Bhatia SN. Three-dimensional tissue fabrication. *Adv. Drug Deliv. Rev.* 56(11), 1635–1647 (2004).

6. Fernandez P, Deguette A, Pothuaud L, Belleannee G, Coste P, Bordenave L. Quality control assessment of ePTFE precoating procedure for *in vitro* endothelial cell seeding. *Biomaterials* 26(24), 5042–5047 (2005).
7. Bordenave L, Menu P, Baquey C. Developments towards tissue-engineered, small-diameter arterial substitutes. *Expert Rev. Med. Devices* 5, 337–347 (2008).
8. Weinberg CB, Bell E, A blood vessel modelconstructed from collagen and cultured vascular cells. *Science* 231(4736), 397–400 (1986).
9. Sultana T, Amirian J, Park C, Lee SJ, Lee BT. Preparation and characterization of polycaprolactone–polyethylene glycol methyl ether and polycaprolactone–chitosan electrospun mats potential for vascular tissue engineering. *J. Biomater. Appl.* 32(5), 648–662 (2017).

•• **Demonstrated the fabrication of polycaprolactone/chitosan nanofibers for vascular tissue engineering.**

10. Levenberg S. Engineering blood vessels from stem cells: recent advances and applications. *Curr. Opin. Biotechnol.* 16(5), 516–523 (2005).
11. Ventola CL. Medical applications for 3D printing: current and projected uses. *Pharm. Ther.* 39, 704–711 (2014).
12. Sung HJ, Meredith C, Johnson C, Galis ZS. The effect of scaffold degradation rate on three-dimensional cell growth and angiogenesis. *Biomaterials* 25, 5735–5742 (2004).
13. Ji X, Yang W, Wang T *et al.* Coaxially electrospun core/shell structured poly(L-lactide) acid/chitosan nanofibers for potential drug carrier in tissue engineering. *J. Biomed. Nanotechnol.* 9, 1672–1678 (2013).
14. Uchida T, Ikeda S, Oura H *et al.* Development of biodegradable scaffolds based on patient-specific arterial configuration. *J. Biotechnol.* 133(2), 213–218 (2008).
15. Marion MM, Mao JJ. Mesenchymal stem cells and tissue engineering. *Methods Enzymol.* 420, 339–361 (2006).

• **Study reporting the importance of mesenchymal stem cells for the regeneration of diseased or damaged tissues and organs.**

16. Tamama K, Sen CK, Wells A. Differentiation of bone marrow mesenchymal stem cells into the smooth muscle lineage by blocking ERK/MAPK signaling pathway. *Stem Cells Dev.* 17(5), 897–908 (2008).
17. Xin X, Hussain M, Mao JJ. Continuing differentiation of human mesenchymal stem cells and induced chondrogenic and osteogenic lineages in electrospun PLGA nanofiber scaffold. *Biomaterials* 28(2), 316–325 (2007).
18. Ball SG, Shuttleworth CA, Kielty CM. Vascular endothelial growth factor can signal through platelet-derived growth factor receptors. *J. Cell Biol.* 177(3), 489–500 (2007).

•• **Study VEGF induces the cells upon differentiation into vascular cell types in vascular tissue engineering.**

19. Krawiec JT, Vorp DA. Adult stem cell-based tissue engineered blood vessels: a review. *Biomaterials* 33(12), 3388–3400 (2012).
20. Mathapati S, Bishi DK, Venugopal JR *et al*. Nanofibers coated on acellular tissue-engineered bovine pericardium supports differentiation of mesenchymal stem cells into endothelial cells for tissue engineering. *Nanomedicine* 9(5), 623–634 (2014).

•• **Study nanofibers spun on acellular bovine pericardium for the culture of mesenchymal stem cells for vascular tissue engineering.**

21. Venugopal JR, Ramakrishna S. Nanotechnology: 21st century revolution in restorative healthcare. *Nanomedicine* 11(12), 1511–1513 (2016).

• **Importance of electrospun nanofibers for healthcare applications in the 21st century.**

22. Yang W, He N, Li Z. Rapamycin release study of porous poly(L-lactic acid) scaffolds, prepared via coaxial electrospinning. *J. Nanosci. Nanotechnol.* 16, 9404–9412 (2016).
23. Yukseloglu SM, Sokmen N, Canoglu S. Biomaterial applications of silk fibroin electrospun nanofibres. *Microelectron. Eng.* 146, 43–47 (2015).
24. Liao I, Chew S, Leong K. Aligned core–shell nanofibers delivering bioactive proteins. *Nanomedicine* 1(4), 465–471 (2006).
25. Ghasemi-Mobarakeh L, Prabhakaran MP, Morshed M, Nasr-Esfahani MH, Ramakrishna S. Electrospun poly(ϵ-caprolactone)/gelatin nanofibrous scaffolds for nerve tissue engineering. *Biomaterials* 29(34), 4532–4539 (2008).
26. Sarkar S, Lee GY, Wong JY, Desai TA. Development and characterization of a porous micro-patterned scaffold for vascular tissue engineering applications. *Biomaterials* 27(27), 4775–4782 (2006).
27. Wang T, Ji X, Jin L *et al.* Fabrication and characterization of heparin-grafted poly-L-lactic acid–chitosan core–shell nanofibers scaffold for vascular gasket. *ACS Appl. Mater. Interfaces* 5, 3757–3763 (2013).
28. Yang W, He N, Juan Fu Z, Ji X. Preparation of porous core–shell poly l-lactic acid/polyethylene glycol superfine fibres containing drug. *J. Nanosci. Nanotechnol.* 15(12), 9911–9917 (2015).
29. Shalumon KT, Deepthi S, Anupama MS, Nair SV, Jayakumar R, Chennazhi KP. Fabrication of poly (l-lactic acid)/gelatin composite tubular scaffolds for vascular tissue engineering. *Int. J. Biol. Macromol.* 72, 1048–1055 (2015).
30. Heydarkhan-Hagvall S, Schenke-Layland K, Dhanasopon AP *et al.* Three-dimensional electrospun ECM-based hybrid scaffolds for cardiovascular tissue engineering. *Biomaterials* 29(19), 2907–2914 (2008).
31. Fukunishi T, Best CA, Sugiura T *et al.* Tissue-engineered small diameter arterial vascular grafts from cell-free nanofiber PCL/chitosan scaffolds in a sheep model. *PLoS ONE* 11(7), 1–15 (2016).
32. Garcia-Fuentes M, Meinel AJ, Hilbe M, Meinel L, Merkle HP. Silk fibroin/hyaluronan scaffolds for human mesenchymal stem cell culture in tissue engineering. *Biomaterials* 30(28), 5068–5076 (2009).

33. Zhou J, Cao C, Ma X, Lin J. Electrospinning of silk fibroin and collagen for vascular tissue engineering. *Int. J. Biol. Macromol.* 47(4), 514–519 (2010).
34. Liu H, Li X, Zhou G, Fan H, Fan Y. Electrospun sulfated silk fibroin nanofibrous scaffolds for vascular tissue engineering. *Biomaterials* 32(15), 3784–3793 (2011).
35. Zhang X, Baughman CB, Kaplan DL. *In vitro* evaluation of electrospun silk fibroin scaffolds for vascular cell growth. *Biomaterials* 29(14), 2217–2227 (2008).
36. Tian L, Prabhakaran MP, Ding X, Kai D, Ramakrishna S. Emulsion electrospun vascular endothelial growth factor encapsulated poly(l-lactic acid-*co*-ϵ-caprolactone) nanofibers for sustained release in cardiac tissue engineering. *J. Mater. Sci.* 47(7), 3272–3281 (2012).

•• **Demonstrates VEGF-loaded poly(l-lactic acid-*co*-ϵ-caprolactone; [PLACL]) electrospun nanofibers showing decreased fiber diameter.**

37. Gosline JM, Guerette PA, Ortlepp CS, Savage KN. The mechanical design of spider silks: from fibroin sequence to mechanical function. *J. Exp. Biol.* 202, 3295–3303 (1999).
38. Mrówczyński W, Mugnai D, de Valence S *et al*. Porcine carotid artery replacement with biodegradable electrospun poly-e-caprolactone vascular prosthesis. *J. Vasc. Surg.* 59(1), 210–219 (2014).
39. Kai D, Prabhakaran MP, Jin G, Tian L, Ramakrishna S. Potential of VEGF-encapsulated electrospun nanofibers for *in vitro* cardiomyogenic differentiation of human mesenchymal stem cells. *J. Tissue Eng. Regen. Med.* 11(4), 1002–1010 (2015).
40. Nottelet B, Pektok E, Mandracchia D, Walpoth B, Gurny R, Möller M. Factorial design optimization and *in vivo* feasibility of poly(epsilon-caprolactone)-micro- and nanofiber-based small diameter vascular grafts. *J. Biomed. Mater. Res. A* 89(4), 865–875 (2009).

• **Discusses the feasibility of polycaprolactone nanofibers for vascular grafts.**

41. Gandhimathi C, Venugopal JR, Tham AY, Ramakrishna S, Kumar SD. Biomimetic hybrid nanofibrous substrates for mesenchymal stem cells differentiation into osteogenic cells. *Mater. Sci. Eng. C* 49, 776–785 (2015).
42. Pereira IHL, Ayres E, Averous L *et al*. Elaboration and characterization of coaxial electrospun poly(ϵ-caprolactone)/gelatin nanofibers for biomedical applications. *Adv. Polym. Technol.* 33(S1), doi:10.1002/adv.21475 (2014).
43. Ravichandran R, Sridhar R, Venugopal JR, Sundarrajan S, Mukherjee S, Ramakrishna S. Gold nanoparticle loaded hybrid nanofibers for cardiogenic differentiation of stem cells for infarcted myocardium regeneration. *Macromol. Biosci.* 14(4), 515–525 (2014).
44. Soffer L, Wang X, Zhang X *et al.* Silk-based electrospun tubular scaffolds for tissue-engineered vascular grafts. *J. Biomater. Sci. Polym. Ed.* 19, 653–664 (2008).
45. Gautam S, Dinda AK, Mishra NC. Fabrication and characterization of PCL/gelatin composite nanofibrous scaffold for tissue engineering applications by electrospinning method. *Mater. Sci. Eng. C* 33(3), 1228–1235 (2013).
46. Huang NF, Li S. Mesenchymal stem cells for vascular regeneration. *Regen. Med.* 3(6), 877–892 (2008).
47. Fukata Y, Kaibuchi K, Amano M, Kaibuchi K. Rho-Rho-kinase pathway in smooth muscle contraction and cytoskeletal reorganization of non-muscle cells. *Trends Pharmacol. Sci.* 22(1), 32–39 (2001).
48. Zhou R, Zhu L, Fu S, Qian Y, Wang D, Wang C. Small diameter blood vessels bioengineered from human adipose-derived stem cells. *Sci. Rep.* 6, 1–11 (2016).